

Can the haemorrhagic component of heparin be identified?

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Can the Haemorrhagic Component of Heparin Be Identified? Or an Attempt at Clean Thinking on a Dirty Drug

Abstract

Heparin consists of different classes of molecules. We distinguish below-critical-chain length heparin (BCLM, MW < 5,400), with only anti-factor Xa activity and above-critical-chain length material (ACLM, MW > 5,400) with both antithrombin and anti-factor Xa activity. In this article we introduce a division within the ACLM fraction, between extra large material (MW > 8,000) and ACLM-low (MW 5,400–8,000). Extra large material is abundantly present in unfractionated heparin but is rare in low-molecular-weight (LMW) heparins. We noted that injection of an LMW heparin causes 5- to 10-fold higher plasma levels of ACLM than injection of a clinically equivalent dose of unfractionated heparin (UFH) and proportionally higher inhibitions of the clotting system. So with LMW heparin one can afford higher levels of anticoagulation than with UFH at a lower risk of bleeding. We surmise that this is caused by the virtual absence of the (haemorrhagic) extra-large-molecular-weight fraction from LMW heparins. A laboratory artefact, i.e. the absence of Ca^{2+} in the anti-factor Xa tests, makes that heparin mixtures that lack extra large heparin molecules show a (spuriously) high ratio of anti-factor Xa activity over anti-thrombin activity. So the correlation between a high aXa/alla ratio and a favourable ratio of antithrombotic effect over bleeding is not necessarily caused by the presence of BCLM. In fact BCLM is a poor anticoagulant; in mixtures of ACLM and BCLM, ACLM causes by far the larger part of the anticoagulant effect. We surmise that the LMW fraction of ACLM is the active anticoagulant component in any heparin preparation and, isolated, would make a proper third-generation heparin.

A Dirty Drug?

We may consider ourselves and our patients lucky that the classical unfractionated heparin (UFH) was introduced and accepted

before drug regulation took its present magisterial proportions. Today it would not stand a chance to pass the authorities, because it is notoriously ill-defined and heterogeneous. Only a limited part (30–45%) of all molecules

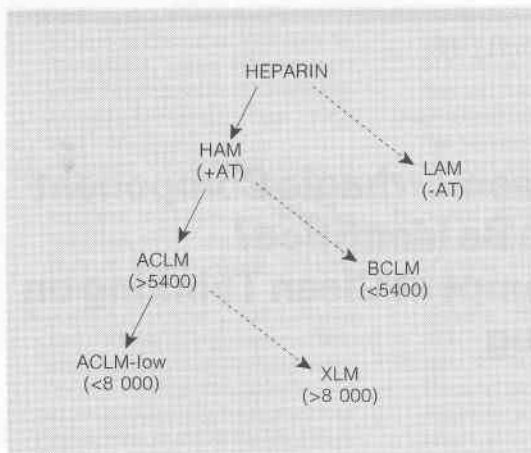


Fig. 1. A classification of heparin subspecies.

contain the pentasaccharide that endows the molecule with the property to bind with high affinity to anti-thrombin (AT) and thereby to catalyse the interaction of that inhibitor with clotting enzymes. This part we call the high-affinity material (HAM) [1–3]. The remaining low-affinity material (LAM) consists of molecules that do not contribute directly and significantly to the overall antithrombotic action [4–6; see also below].

Low-molecular-weight heparins (LMWHs) are prepared from UFH by reducing the molecules to about one half or one third of their original sizes. During this process, the active pentasaccharide is sometimes cleft, which explains that LMWHs contain less HAM than the original UFH and that the percentage of HAM decreases with decreasing molecular weight (MW) [7]. The decreasing MW brings about a spectacular increase in bioavailability after subcutaneous injection [8, 9; see also ref. 10, in which many others are cited] and produces a new fraction: heparin molecules that do contain an intact pentasaccharide but that are shorter than 17 monosaccharide units. Such molecules will no longer catalyse throm-

bin inhibition but will retain their activity towards factor Xa [11–13]. This divides HAM in two species: ACLM, i.e. above-critical-length material, that accelerates the inhibition of both thrombin and factor Xa, and BCLM, below-critical-length material, that affects factor Xa inhibition only (fig. 1).

As we will discuss below, we surmise that essential differences exist between the ACLM contained in LMWH and that in UFH. We therefore want to distinguish as a separate class those ACLM molecules that abound in UFH but are rare in LMWHs, i.e. the higher-MW fraction of ACLM. The boundary between the higher- and the lower-MW fraction within ACLM is arbitrary for the moment, it may be situated anywhere between MW 8,000 and MW 12,000. We do not even mean to suggest that there are only two types of ACLM. There may be a sharp boundary at an as yet unknown MW, or there may be a gradual shift in properties going from low to high MW. For the moment, we use 8,000 as an arbitrary boundary. Where it is necessary to make the distinction, we will indicate the 5,400–8,000 MW fraction of ACLM as ‘ACLM-low’. The high-MW fraction we call extra large ACLM (XLM) (fig. 1).

In LMWHs the ‘therapeutic gap’, i.e. the range of concentrations in which the antithrombotic profit outweighs the harm of the bleeding tendency induced, is larger than in UFH [14–16; other examples in ref. 10]. This indicates that the properties to decrease thrombosis and to increase bleeding are not equally distributed over heparin fractions of different MW.

If it would be possible to identify a subspecies that carries the anticoagulant activity and another that is responsible for the bleeding activity, then it would be possible to identify the better LMW heparins as those with the maximum amount of the ‘right’ subspecies and the minimum of the ‘wrong’ one. Even

more interesting, identification of the 'right' subspecies would provide a guideline to prepare a superior, third-generation, heparin. The problem is that the fractions are not available for human or animal experimentation. UFH is a mixture of ACLM and XLM with negligible amounts of BCLM. LMWHs are varying mixtures of BCLM, ACLM-low and some XLM, the composition of the mixture determining its therapeutic gap [17]. Only isolated BCLM is available in the form of the synthetic AT-binding pentasaccharide [18, 19]. All other heparin preparations are mixtures; one could paraphrase Ephraim Racker's advice 'Don't waste clean thinking on dirty enzymes' and wait until purer heparin fractions are available. In our opinion, the existing data already allow us to make an educated guess of what the antithrombotic- and the haemorrhagic subspecies are.

We are aware of the fact that, probably, the antithrombotic activity of heparin is due to more than its AT-mediated direct anticoagulant properties. Heparin e.g. causes the release of TFPI from the vessel wall and thus provokes the appearance of another anticoagulant. It may well carry still other, as yet unrecognised, antithrombotic actions. Yet the anticoagulant effect of heparin must be considered to be its main antithrombotic property [4–6], and our discussion will be primarily in these terms.

Clean Thinking?

When LMWH was introduced, it was observed that it causes less bleeding than UFH at the same anticoagulant activity. Also the anti-factor Xa activity (aXa) of LMWH appeared higher than that of UFH at identical anti-thrombin activity (alla). So a high aXa/alla ratio indicates favourable antithrombotic properties in a heparin [see also ref. 10]. The

high aXa/alla ratios were attributed to the presence of BCLM, the species without anti-thrombin activity. As a logical consequence, it was thought that BCLM carries the antithrombotic activity whereas the higher MW fractions, i.e. ACLM, cause haemorrhage. On closer observation of the evidence, it appears that this conclusion is not justified. The favourable properties of a heparin appear to reside in the LMW portion of the ACLM fraction, the unfavourable ones in the XLM fraction and the BCLM fraction does not carry much weight.

In fact there is no close correlation between aXa activity and BCLM concentration! The high aXa/alla ratio in LMWHs is primarily due to the fact that the lower MW fractions of ACLM do not need Ca^{2+} ions for the full expression of their aXa action, whereas the higher MW fractions (i.e. XLM) do [20]. This, in combination with the fact that all current aXa determinations are carried out in the absence of Ca^{2+} ions makes that the aXa action of XLM is systematically underestimated. Because the international standard heparin is of the XLM type, this effect causes a spurious overestimation of the aXa activity of ACLM-low and BCLM. A high aXa/alla ratio therefore is indicative of the absence of XLM as much as of the presence of BCLM. (The problem of overestimation of aXa activity in LMWHs is *not* solved by the adoption of the LMWH standard; the activity of this standard is itself determined relative to the international standard in tests without Ca^{2+} ions, and so is itself overestimated.)

In fact, the aXa activity is a severely compromised indicator of heparin activity. Under the usual laboratory conditions, it measures all high-affinity material, BCLM and ACLM alike. Any given aXa activity can be caused by an infinite number of mixtures of BCLM and ACLM. Yet the antithrombotic properties of these fractions are entirely different. The

LMWHs Are Not Likely to Act via Their Lowest MW Fraction (BCLM)

common practice to discuss heparin pharmacology in terms of aXa activity only [for examples, see the overview in ref. 10] therefore is a hurdle to our understanding of heparin pharmacology rather than a help.

There is an alternative though. Heparin pharmacology should be based on the plasma levels of the ACLM and BCLM fractions instead of the indiscriminating aXa activity. Indeed one can use the available anti-factor Xa and anti-thrombin determinations to calculate the levels of ACLM and BCLM that are present in a given plasma sample [8, 21, 22]. The procedure is simple. The total HAM (= ACLM + BCLM) content can be obtained from the aXa figure, whereas the ACLM concentration can be calculated from the alla activity. The BCLM figure is then obtained by subtraction. Once we can determine the concentration of the heparin subspecies that circulate in plasma, we can determine what their contribution is to the overall anticoagulant effect.

It then appears that BCLM contributes only little to the anticoagulant action of current LMWH preparations [23–27]. In an LMWH preparation that contains both ACLM and BCLM, it is the ACLM that carries by far the larger part of the anticoagulant action, both in vitro with the fractions added to normal plasma and in vivo [8]. This evidence will be presented in detail in the following paragraph.

Yet, if BCLM is not the fraction that endows LMWH with its favourable properties, what fraction is? What causes the increase of the therapeutic gap together with the increase of the aXa/alla ratio? In the paragraph to follow we will show evidence that it is the lack of the XLM fraction rather than the presence of BCLM that makes LMWH better than UFH.

There exists a large amount of literature on the relative importance of alla and aXa activity for the antithrombotic action of heparins. The scales seem to tilt slightly in favour of alla action [10, 28] but it is clear that the outcome depends on the thrombosis model that is used [29, 30]. In the light of the discussion in the previous paragraph one may well ask whether the question posed in this way makes sense and if not the more important question would be whether the antithrombotic effect is due to ACLM or to BCLM.

Indeed studies in which alla and aXa activities are correlated with thrombosis scores are extremely difficult to interpret. There necessarily is a high degree of linkage between the two activities as such, because they are for an important part carried by the same molecule (ACLM) and if they are not, they are injected together because current LMWHs are a mixture of ACLM and BCLM. This high covariation makes interpretation of the outcome extremely risky. Differences in experimental accuracy may also play a role. An existing correlation may be blurred by random experimental error in the laboratory determinations. If the error were larger in the antithrombin than in the anti-factor Xa measurements then this might show up as a difference in the significance of the correlation. Also the spurious increase of the aXa/alla ratio in ACLM-low molecules caused by the Ca^{2+} effect discussed above [20] will show up as an antithrombotic effect of aXa activity even if ACLM-low and not BCLM is the active molecule.

In fact there are two different questions. One is whether aXa or aXa action per se is antithrombotic. The other is whether ACLM or BCLM is the antithrombotic fraction if LMWH. The first one is easily solved by administration of antithrombotics that affect

one of the two clotting enzymes only. There is no doubt that pure antithrombin action [dermatan sulfate; 31] as well as pure anti-factor Xa action [pentasaccharide; 18, 19] are both antithrombotic. This probably reflects the fact that any drug that diminishes thrombin in clotting plasma will be antithrombotic, independent of the way in which the effect is achieved.

The next question is whether ACLM or BCLM is the active fraction in LMWH. On the basis of the high aXa/alla ratio in LMWHs, many authors jumped to the conclusion that BCLM must be the active molecule. This thought was probably at the basis of the efforts to try the active pentasaccharide and its congeners as antithrombotic drugs. Several lines of evidence have been produced, however, that make us believe that the role of BCLM must be rather modest.

In vitro, heparins invariably cause an important decrease of the area under the thrombin generation curve [the thrombin potential; 32, 33]. This can be brought about by an increase of thrombin inactivation (as in the case of dermatan sulfate) or by an inhibition of prothrombin conversion [as in the case of pentasaccharide; 26] or by a combination of both. Via a method that allows to determine the relative contribution of both processes [34], it could be determined that thrombin inactivation is the more important mechanism via which heparin acts [23–27]. Insofar as prothrombin activation is inhibited, this is rather by inhibition of thrombin-mediated feedback activation of factors V and/or VIII than by heparin-induced inhibition of factor Xa [23, 27, 35].

In vitro experiments of prothrombinase inhibition in flow systems on macroscopic surfaces corroborate this conclusion. These experiments are designed to mimick the in vivo situation in which prothrombinase (i.e. the complex of factors Xa and Va absorbed on a

procoagulant phospholipid surface) is localised on activated platelets adhered to a wound or a thrombus surface. In this situation the rate of thrombin production is determined by the rate at which prothrombin is delivered to the surface by diffusion [36]. Inhibition of prothrombinase is of no consequence unless there is so few prothrombinase left on the surface that its capacity to convert prothrombin becomes lower than the velocity with which prothrombin can arrive there by diffusion (less than about 1 per μm^2 , which is less than 0.1% of the available binding sites on an activated platelet). If that situation occurs, however, then prothrombinase is always maximally saturated with prothrombin, and we could show that such occupied complexes are immune to inhibition by AT-heparin [37]. So AT-heparin can inhibit those prothrombinase complexes that are not producing thrombin anyhow, but not those that are actively converting prothrombin into thrombin [37] (side step for the connoisseur: the observed K_m of prothrombinase is indeed very low, around 2 mM). Preliminary results show that some kinds of heparin, independent of MW are more efficient inhibitors at macroscopic surfaces than others. Anyhow, in vitro experiments predict that high concentrations of aXa activity will be necessary to inhibit prothrombinase effectively, so that high proportions of BCLM [such as present only in certain types of LMWH such as enoxaparin; 25] should be present if BCLM is to contribute to the anticoagulant effect.

In vivo observations are in complete agreement with these predictions. Plasma samples obtained after injection of UFH and of enoxaparin and tested for their ACLM and BCLM content as well as thrombin generation, show that the ACLM concentration determines the anticoagulant effect and that the contribution of BCLM is negligible [8].

The relative unimportance of the BCLM fraction in current LMWHs does by no means

imply that BCLM when administered *without* (or with minimal amounts of) ACLM could not have anticoagulant and antithrombotic properties [18, 19]. BCLM attacks factor Xa, which is a constituent of prothrombinase. An attack on factor Xa will in the end impede prothrombin activation. Yin et al. [38, 39] coined the very logical idea that it is more effective to inhibit the thrombin-producing enzyme than to remove thrombin itself. That it is wiser to close the tap than to mop the floor. Yet, logical as it may seem, the reasoning does not hold because inhibiting factor Xa is an inefficient way to inhibit prothrombinase. Prothrombinase is a complex of factors Xa and Va assembled on a phospholipid surface [40, see further ref. 41]. Its concentration depends upon the concentration of its constituents. In clotting plasma, factor Va is the rate-limiting component and factor Xa is present in excess. (The plasma concentration of factor X is around 200 nM, that of factor V is 20 nM. In the course of events that leads to clotting, factor X is activated first and factor V later) [see further ref. 41]. Even if factor Xa is inhibited by 50%, prothrombinase activity is still hardly diminished. Inhibition of factor Xa for 90% inhibits prothrombin conversion by 50%. Compared to inhibition of thrombin activity, inhibition of factor Xa is an inefficient means to influence the blood-clotting process. If both aXa and alla activities are present simultaneously, then the aXa activity will remain in the shadow unless the ACLM content becomes very low. If only the aXa activity is there, as in the synthetic AT-binding pentasaccharides, then of course thrombin generation can be inhibited via the inhibition of prothrombinase [26].

Those that want to obtain anticoagulant action via the inhibition of prothrombin conversion (i.e. by anti-factor Xa activity) will therefore probably have to go to relatively high inhibitions of factor Xa.

In summary: on a molar basis, ACLM is a tenfold more effective anticoagulant than BCLM is. Only if the BCLM molecules outnumber the ACLM by a factor of ten or more, will the BCLM contribute significantly to the overall anticoagulant effect in plasma. In practice the admixture of BCLM to ACLM will hardly contribute to the anticoagulant properties of the latter. The admixture of BCLM to ACLM as we observe it in the current, clinically used LMWH preparations will not or hardly contribute to anticoagulant action, but it does contribute to the aXa activity and so is responsible for much of the confusion that exists in the laboratory assessment of heparin activity.

LMWHs Are Likely to Lack a Haemorrhagic Component Present in Unfractionated Heparin

If the difference between LMWH and UFH is not likely to reside in the presence of the BCLM fraction, then it must reside in the ACLM fraction. Indeed there are large differences in the pharmacokinetics of the ACLM from LMWH and from UFH. We found that the bioavailability of the ACLM fraction of a LMWH after subcutaneous injection is 6–8 times higher than that of UFH [8]. This finding is backed up by a large number of studies in which the bioavailability of LMWHs and of UFH has been compared without making the distinction between ACLM and BCLM [see ref. 10 for a review]. What can be the cause of this shift in bioavailability with the MW distribution? One possibility is that only ACLM-low is found back in the blood stream and that XLM does not appear at all. The low bioavailability of UFH, would then reflect its low proportion of ACLM-low. This is corroborated by the finding of an identical apparent pharmacological half-life time for the ACLM after subcutaneous injection of either LMWH

or UFH [8]. It is well known that the half-life time of a heparin varies strongly with the molecular size [10, 42]. Identical half-life times suggest that the MW distribution of the ACLM in the blood after injection of UFH is similar to that after injection of LMWH.

If indeed only ACLM-low is the bioavailable species and if XLM is not to be found in the blood stream, this might be explained in two ways: either XLM does not travel from the injection site to the blood stream or XLM disappears so quickly from the blood that it is never observed. It is quite conceivable that it sticks to the vessel wall immediately [43] and in this way affects hemostasis locally.

One might consider to administer UFH in doses that are seven times higher than the usual ones (e.g. 35,000 IU twice daily) so as to obtain the same plasma levels of ACLM as in a prophylactic regimen with a LMWH. Without any doubt, judging from clinical experience, this would cause a sizeable increase of the bleeding frequency. So, although the amount of anticoagulant material in the blood stream would be the same, the bleeding risk would be much increased. This suggests that there is haemorrhagic material contained in UFH but not (or much less) in LMWH. The obvious candidate is XLM and/or the LAM fraction of the same size.

From the above we surmise that in any current heparin preparation, the ACLM-low fraction, i.e. the high affinity fraction of between 5,400 and 8,000 MW, is the one that brings about anticoagulation at a low bleeding risk. Fractions with a MW <5,400, as discussed above, are not likely to be the active component in current LMWHs. Fractions above a certain limit (i.e. XLM) may not reach the blood stream or disappear very fast, and probably have haemorrhagic properties.

Again we stress that 8,000 as a lower MW boundary for XLM is arbitrary. It has not been determined experimentally. Its actual

value can be determined by measuring the MW distribution of the heparin fractions that circulate after subcutaneous injection of UFH or LMWH.

The ACLM-low fraction makes a good candidate for the ideal heparin. It will in all probability combine a high anticoagulant potency (and therefore a low IC₅₀ in venous thrombosis models) with reliable and high bioavailability at minimal haemorrhagic risk. Unlike with UFH, the haemorrhagic complications will probably be related to the degree of anticoagulation obtained. The plasma levels of this material will be easy to determine via its aXa activity but, contrary to the present LMWHs that contain BCLM, the outcome will be readily interpretable. In essence, this material is good old UFH with a high bioavailability and without the high-MW molecules that are likely to cause haemorrhage.

A drawback of this fraction might be that, unlike BCLM, it is neutralised by heparin-binding material from activated platelets. Recent work from the group of Boneu [29, 30] has shown that (in rabbits) in venous models the antithrombotic activity seems to correlate with the AT activity of the material injected, whereas in arterial models the aXa activity seems to gain in importance. Although the data have to be interpreted with caution (see above), this reminds us that thrombosis is sufficiently diverse to need a diverse therapeutic approach. The above-defined heparin fraction is therefore not necessarily optimal for both venous and arterial thrombosis. It is an educated guess that it will be the useful heparin for the prevention of venous thrombosis. It will be neutralised, however, by platelet factor 4 from activated platelets. BCLM is not, or much less, so one can imagine that a pure BCLM fraction is the drug of choice in the prevention of growth of arterial thrombi [44, 45].

It seems that at this moment a pure BCLM is being developed for clinical usage. It is our

guess that ACLM-low without BCLM and without XLM is at least as interesting a product and merits to be prepared and tested in a clinical trial.

What Use Is the LAM?

It has been demonstrated that the LAM fraction in itself does not have significant antithrombotic properties but that it can enhance the antithrombotic properties of HAM [4–6]. One can imagine the following mechanisms: (a) LAM has AT activity via its action on heparin cofactor II [46]. For this action to be significant, concentrations of heparin are needed that are $10\text{--}20 \times$ higher than those generally used, so this effect will be negligible in practice [47]. (b) LAM may release TFPI from the vessel wall. If TFPI is washed from the vessel wall by a mechanism that is independent of AT-binding then LAM and HAM of equal size and charge will not differ in this respect. The contribution to the antithrombotic effect is unknown. (c) LAM will displace HAM from non-specific binding sites on cells and plasma proteins, in this way setting active heparin molecules free for their specific interaction with AT [48, 49]. This phenomenon is responsible for the fact that in plasma heparin action is proportional to AT concentration, even though AT is present in excess over the concentration of heparin, so that one might think that all heparin would be bound to AT (to give an approximative idea of the molar concentrations involved: a plasma concentration of 1 IU/ml of UFH represents about $5 \mu\text{g/ml}$ of crude material, 35% of which is HAM. At an assumed MW of around 10,000, this represents $0.1\text{--}0.2 \mu\text{M}$ of active material. AT III is present at a concentration of $2\text{--}3 \mu\text{M}$).

None of these functions of LAM has been shown to be essential for the anticoagulant action of heparin in patients, although they

may contribute to it. On the other hand, a contribution of LAM to the bleeding effect has been observed [50].

It is logical to think that antigenic- and toxic effects are a function of specific oligosaccharide sequences within the heparin molecule, which will automatically be more abundant in higher-molecular-weight heparins. We conclude that, as far as we can reason, LAM does not carry a known useful function, that it may contribute to undesired side effects of heparin and that larger LAM molecules will be worse in this respect than smaller ones.

Retrospective and Future

In retrospect, those who claimed that a high aXa activity makes a good heparin were right, be it for reasons that they did not suspect. It is not because the molecules without AT activity (BCLM), have particularly good properties but, more probably than not, because the ACLM-low fraction, which by favour of its high anticoagulant activity, its high bioavailability and long half-life time is the better component of heparin, is also the fraction that is overestimated in the usual aXa-tests. The XLM fraction, that is likely to cause bleeding, is not overestimated.

Of course the hypotheses forwarded here require more experimental backup. A relatively simple but revolutionary experiment would be to isolate XLM and show that it is an inefficient antithrombotic that causes bleeding. This expected outcome would make further use of UFH rather questionable. A more scholarly approach would be to determine the MW distribution of heparin fractions found in circulation after subcutaneous injection of UFH. Anyhow it is clear from the present considerations that the presently available LMWHs are a step forward compared to UFH but may still be improved considerably.

References

- Andersson LO, Barrowcliffe TW, Holmer E, Johnson EA, Sims GEC: Anticoagulant properties of heparin fractionated by affinity chromatography on matrix-bound antithrombin III and by gel filtration. *Thromb Res* 1976;9:575-583.
- Höök M, Björk I, Hopwood J, Lindahl U: Anticoagulant activity of heparin: Separation of high-activity and low-activity heparin species by affinity chromatography on immobilized antithrombin. *FEBS Lett* 1976;66:90-93.
- Denton J, Lewis WE, Nieduszynski IA, Phelps CF: Fractionation of heparin using antithrombin III reversibly bound to concanavalin A-Sepharose. *Anal Biochem* 1981;118:388-391.
- Barrowcliffe TW, Merton RE, Havercroft SJ, Thunberg L, Lindahl U, Thomas DP: Low-affinity heparin potentiates the action of high-affinity heparin oligosaccharides. *Thromb Res* 1984;34:125-134.
- Merton RE, Thomas DP, Havercroft SJ, Barrowcliffe TW, Lindahl U: High and low affinity heparin compared with unfractionated heparin as antithrombotic drugs. *Thromb Haemost* 1984;51:254-256.
- Zammit A, Dawes J: Low-affinity material does not contribute to the antithrombotic activity of organan (Org 10172) in human plasma. *Thromb Haemost* 1994;71:759-767.
- Bendetowicz AV, Pacaud E, Béguin S, Uzan A, Hemker HC: On the relationship between molecular mass and anticoagulant activity of a low molecular weight heparin (Enoxaparin). *Thromb Haemost* 1992;67:556-562.
- Bendetowicz AV, Béguin S, Caplain H, Hemker HC: Pharmacokinetics and pharmacodynamics of a low molecular weight heparin (Enoxaparin) after subcutaneous injection, comparison with unfractionated heparin. A three-way cross-over study in human volunteers. *Thromb Haemost* 1994;71:305-313.
- Briant L, Caranobe C, Saivin S, Sie P, Bayrou B, Houin G, Boneu B: Unfractionated heparin and CY 216: Pharmacokinetics and bio-availabilities of the antifactor Xa and IIa Effects after intravenous and subcutaneous injection in the rabbit. *Thromb Haemost* 1989;61:348-353.
- Barrowcliffe TW, Johnson EA, Thomas DP: Low molecular weight heparin. Chichester, Wiley, 1992.
- Andersson LO, Barrowcliffe TW, Holmer E, Johnson EA, Soderstrom G: Molecular weight dependency of the heparin. Potentiated inhibition of thrombin and activated factor X. Effect of heparin neutralization in plasma. *Thromb Res* 1979;15:531-541.
- Lane DA, MacGregor IR, VanRoss M, Cella G, Kakkar VV: Molecular weight dependence of the anticoagulant properties of heparin: Intravenous and subcutaneous administration of fractionated heparins to man. *Thromb Res* 1979;16:651-662.
- Lane DA, Denton J, Flynn AM, Thunberg L, Lindahl U: Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem J* 1984;218:725-732.
- Kakkar VV, Cohen AT, Edmonson RA, Phillips MJ, Cooper DJ, Das SK, Maher KT, Sanderson RM, Ward VP, Kakkar S: Low molecular weight versus standard heparin for prevention of venous thromboembolism after major abdominal surgery. *Lancet* 1993;341:259-265.
- Kakkar VV: Prevention and management of venous thrombosis. *Br Med Bull* 1994;50:871-903.
- Kakkar VV: Efficacy and safety of clivarin registered and other LMWHs in general surgery: A Meta-analysis blood. *Coagul Fibrinolysis* 1993;4(suppl 1):S23-S27.
- Cade JF, Buchanan MR, Boneu B, Ockelford P, Carter CJ, Cerskus AL, Hirsh JA: Comparison of the antithrombotic and haemorrhagic effects of low molecular weight heparin fractions: The influence of the method of preparation. *Thromb Res* 1984;35:613-625.
- Walenga J, Petitou M, Lormeau JC, Samama M, Fareed J, Choay J: Antithrombotic activity of a synthetic heparin pentasaccharide in a rabbit stasis thrombosis model using different thrombogenic challenges. *Thromb Res* 1987;46:187-198.
- Thomas DP, Merton RE, Gray E, Barrowcliffe TW: The relative antithrombotic effectiveness of heparin, a low molecular weight heparin, and a pentasaccharide fragment in an animal model. *Thromb Haemost* 1989;61:204-207.
- Hemker HC, Béguin S: The activity of heparin in the presence and absence of Ca^{2+} ions; Why the anti-Xa activity of LMW heparins is about two times overestimated. *Thromb Haemost* 1993;70:717-718.
- Hemker HC, Béguin S, Bendetowicz AV, Wielders S: The determination of the levels of unfractionated heparin and low molecular weight heparins in plasma. Their effect on thrombin-mediated feedback reactions in vivo. Preliminary results on samples after subcutaneous injection. *Haemostasis* 1991;21:258-272.
- Hemker HC, Béguin S: Standard and method independent units for heparin anticoagulant activities. *Thromb Haemost* 1993;70:724-728.
- Béguin S, Lindhout T, Hemker HC: The mode of action of heparin in plasma. *Thromb Haemost* 1988;60:457-462.
- Béguin S, Wielders S, Lormeau JC, Hemker HC: The mode of action of CY 216 and CY 222 in plasma. *Thromb Haemost* 1992;67:33-41.
- Béguin S, Mardiguian J, Lindhout T, Hemker HC: The mode of action of low molecular weight heparin preparation (PK10169) and two of its major components on thrombin generation in plasma. *Thromb Haemost* 1989;61:30-34.
- Béguin S, Choay J, Hemker HC: The action of a synthetic pentasaccharide on thrombin generation in whole plasma. *Thromb Haemost* 1989;61:397-401.

- 27 Hemker HC: The mode of action of heparin in plasma; in Verstraete M, Vermeylen J, Lijnen HR, Arnout J (eds): *Thrombosis and Haemostasis*. Leuven, University Press, 1987, pp 17–36.
- 28 Buchanan MR, Boneu B, Ofosu F, Hirsh J: The relative importance of thrombin inhibition and factor Xa inhibition to the antithrombotic effects of heparin. *Blood* 1985;65: 198–201.
- 29 Amar J, Caranobe C, Sie P, Boneu B: Antithrombotic potencies of heparins in relation to their antifactor Xa and antithrombin activities: An experimental study in two models of thrombosis in the rabbit. *Br J Haematol* 1990;76:94–100.
- 30 Peyrou V, Lormeau JC, Caranobe C, Gabaig AM, Crepon B, Saivin S, Houin G, Sie P, Boneu B: Pharmacological properties of CY 216 and its ACLM and BCLM components in the rabbit. *Thromb Haemost* 1994;72:268–274.
- 31 Merton RE, Thomas DP: Experimental studies on the relative effectiveness of dermatan sulphate and heparin as antithrombotic agents. *Thromb Haemost* 1987;58:839–842.
- 32 Hemker HC, Wielders S, Béguin S: The thrombin potential. A parameter to assess the effect of antithrombotic drugs on thrombin generation; in Bounameaux H, Samama M, ten Cate J.W. (eds): *Fraxiparine, Second International Symposium Recent Pharmacological and Clinical Data*. Stuttgart, Schattauer, 1990, pp 89–101.
- 33 Hemker HC, Wielders S, Kessels H, Béguin S: Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost* 1993;70:617–624.
- 34 Hemker HC, Willems GM, Béguin S: A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost* 1986;56:9–17.
- 35 Ofosu FA, Sie P, Modi GJ, Fernandez F, Buchanan MR, Blajchman MA, Boneu B, Hirsh J: The inhibition of thrombin-dependent positive-feedback reactions is critical to the expression of the anticoagulant effect of heparin. *Biochem J* 1987; 243:579–588.
- 36 Speijer H, Billy D, Willems G, Hemker HC, Lindhout T: Inhibition of prothrombinase by antithrombin-heparin at a macroscopic surface. *Thromb Haemost* 1995;73:648–653.
- 37 Billy D, Speijer H, Willems G, Hemker HC, Lindhout T: Prothrombin activation by prothrombinase in a tubular flow reactor. *J Biol Chem* 1995;270:1029–1034.
- 38 Yin ET, Wessler S, Stoll PJ: Biological properties of the naturally occurring plasma inhibitor to activated factor X. *J Biol Chem* 1971;246: 3703–3711.
- 39 Yin ET, Wessler S, Stoll PJ: Identity of plasma-activated factor X inhibitor with antithrombin III and heparin cofactor. *J Biol Chem* 1971;246: 3712–3719.
- 40 Hemker HC, Esnouf MP, Hemker PW, Swart ACW, MacFarlane RG: Formation of prothrombin converting activity. *Nature* 1967;215:248–251.
- 41 Lindhout T, Hemker HC: Anticoagulant mechanism of action of low molecular weight heparins; in Doutrépuich C (ed): *Low Molecular Weight Heparins in Clinical Practice*. New York, Dekker, 1992, pp 23–50.
- 42 Boneu B, Buchanan MR, Caranobe C, Gabaig AM, Dupouy D, Sie P, Hirsh J: The disappearance of a low molecular weight heparin fraction (CY 216) differs from standard heparin in rabbits. *Thromb Res* 1987; 46:845–853.
- 43 Van-Rijn JL, Trillou M, Mardiguian J, Tobelem G, Caen J: Selective binding of heparins to human endothelial cells. Implications for pharmacokinetics. *Thromb Res* 1987;45: 211–222.
- 44 Béguin S, Lindhout T, Hemker HC: The effect of trace amounts of tissue factor on thrombin generation in platelet rich plasma its inhibition by heparin. *Thromb Haemost* 1989;61: 25–29.
- 45 Bendetowicz AV, Kai H, Knebel R, Caplain H, Hemker HC, Lindhout T, Béguin S: The effect of subcutaneous injection of unfractionated and low molecular weight heparin on thrombin generation in platelet rich plasma. A study in human volunteers. *Thromb Haemost* 1994;72: 705–712.
- 46 Sie P, Petitou M, Lormeau JC, Dupouy D, Boneu B, Choay J: Studies on the structural requirements of heparin for the catalysis of thrombin inhibition by heparin cofactor II. *Biochim Biophys Acta* 1988;966: 188–195.
- 47 Shimotori T, Sakuragawa N: Comparative studies of heparin cofactor activity toward antithrombin III and heparin cofactor II, and antithrombin III affinity between low molecular weight heparin and unfractionated heparin. *Sem Thromb Haemost* 1990;16(suppl):71–76.
- 48 Young E, Prins M, Levine MN, Hirsh J: Heparin binding to plasma proteins, an important mechanism for heparin resistance. *Thromb Haemost* 1992;67:639–643.
- 49 Béguin S, Kessels H, Dol F, Hemker HC: The consumption of antithrombin III during coagulation, its consequences for the calculation of prothrombinase activity and the standardisation of heparin activity. *Thromb Haemost* 1992;68:136–142.
- 50 Ockelford PA, Carter CJ, Cerskus A, Smith CA, Hirsh J: Comparison of the in vivo hemorrhagic and antithrombotic effects of a low antithrombin-III affinity heparin fraction. *Thromb Res* 1982;27:679–690.